

pCRISPomyces-1 and pCRISPomyces-2 Plasmid Assembly Protocol

- 1) Select a 20 nt protospacer of interest. The 3' protospacer adjacent sequence (PAM) must be NGG, where N is any nucleotide. Preference is given to:
 - a. sequences with purines occupying the last four (3') bases of the protospacer.
 - b. sequences on the non-coding strand.
 - c. sequences in which the last 12 nt of protospacer + 3 nt PAM (15 nt total) are unique in the genome (check by BLAST with all four possible NGG sequences).

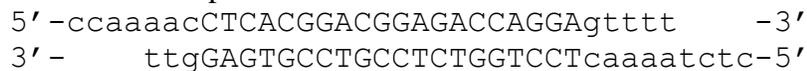
- 2) **For pCRISPomyces-1 only (proceed to step 3 for pCRISPomyces-2):** Design two 32 nt oligonucleotides with the following sequences:

- a. Spacer-for: 5'-ccaaaac N₂₀ gtttt-3'
- b. Spacer-rev: 5'-ctctaaaac N'₂₀ gtt-3'

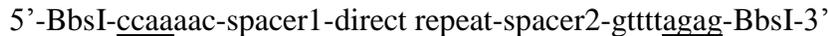
For example, if the spacer sequence is CTCACGGACGGAGACCAGGA, then the two primers are:

- a. Spacer-for: 5'-ccaaaacCTCACGGACGGAGACCAGGAgtttt-3'
- b. Spacer-rev: 5'-ctctaaaacTCCTGGTCTCCGTCCGTGAGgtt-3'

Such that the annealed product will be:



For plasmids with multiple spacers, design a synthetic construct with the following configuration:



Note that the sticky ends for the *BbsI* sites (5' to 3') are CCAA and AGAG (underlined). Proceed to step 4 (for single spacer) or step 5 (for multiple spacers).

- 3) **For pCRISPomyces-2 only:** Design two 24 nt oligonucleotides (4 nt 5' sticky end + 20 nt spacer sequence) with the sticky ends ACGC on the forward primer and AAAC on the reverse primer.

For example, if the spacer sequence is CTCACGGACGGAGACCAGGA, then the two primers are:

- a. Spacer-for: 5'-ACGCCTCACGGACGGAGACCAGGA-3'
- b. Spacer-rev: 5'-AAACTCCTGGTCTCCGTCCGTGAG-3'

Such that the annealed product will be:



For plasmids with two guide RNA cassettes, design a synthetic construct with the following configuration (see example at the end of this document):



Note that the sticky ends for the *BbsI* sites (5' to 3') are ACGC and GTTT (underlined). Proceed to step 4 (for single guide RNA) or step 5 (for multiple guide RNAs).

- 4) For single spacers, anneal spacer oligos as follows:
 - a. Resuspend both oligos to 100µM in water
 - b. Mix 5uL FOR + 5uL REV + 90uL 30mM HEPES, pH 7.8
 - c. Heat to 95°C for 5min, then ramp to 4°C at 0.1°C/sec

- 5) Insert annealed spacer (or dual-spacer/guide synthetic construct) to desired plasmid by Golden Gate assembly.

Golden Gate reaction mixture:

| | | |
|------------------------|----------------------------|--|
| Backbone | X μ L | 100 ng |
| Insert | 0.3 μ L | 3:1 mol ratio or higher (dilute annealed oligos 10-fold) |
| T4 Ligase Buffer (NEB) | 2 μ L | |
| T4 ligase (NEB) | 1 μ L | 400 U/ μ L stock is sufficient; add last |
| BbsI (NEB) | 1 μ L | Stored at -80 °C |
| H ₂ O | <u>Y μL</u> | |
| | 20 μ L | |

Golden Gate Program: 37°C 10 min
16°C 10 min
Goto step 1, 9 times
50°C, 5 min
65°C, 20 min
4°C, forever

- 6) Transform 3 μ L of each reaction to *E. coli* NEB5alpha by heat shock (manufacturer's protocol).
- 7) Plate 10% of recovery culture on selective plates with 10uL of 0.5 M IPTG and 40uL of 20mg/mL Bluo-gal (in DMSO).
- 8) Pick white colonies to selective LB and recover plasmid.
- 9) Meanwhile, PCR amplify two 1 kb homology arms from genomic DNA of the strain of interest to serve as an editing template. [Note: Make sure that the editing template does not include the full protospacer + PAM sequence!] Two options for primer design:
- Design overlaps (20-30 nt) at both ends of both arms and directly perform 3-piece Gibson assembly (1 kb left arm + 1 kb right arm + digested plasmid).
 - Design overlaps (20-30 nt) at the junction of the two arms and *Xba*I cutsites at the opposite ends of the two arms. Then splice the two arms by overlap-extension PCR, and digest and ligate the 2 kb product into the digested plasmid.
- 10) Digest the spacer-containing plasmid (assembled in steps 1-7) with *Xba*I. Dephosphorylate with FastAP (Thermo) or similar to prevent re-ligation.
- 11) Perform the chosen assembly method to insert the 2 kb editing template in your digested, dephosphorylated plasmid.
- 12) Transform assembly product to NEB5alpha competent cells (or similar).
- 13) The next day, pick colonies to LB+apramycin.
- 14) Recover plasmids and confirm by digestion or sequencing.

15) Transform confirmed plasmid to an appropriate conjugation donor strain, and conjugate following standard protocol.

Example synthetic construct for two guide RNAs:

(BbsI-spacer1-gRNAtail-T7term-gapdhp(EL)-spacer2-BbsI)

5'-

GAGACATCTTTGAAGACAA~~acgc~~GTTCTCCGCACTCCCATGAGgttttagagctagaaatagcaagttaaataaggctagtcggt
atcaactgaaaaagtggcaccgagtcggtgcttttttagcataacccttggggcctctaaacgggtcttgaggggtttttggctgctccttcggtcgg
acgtgctctacgggcaccttaccgagccgtcggctgtgacacggacggatcgggcgaactggccgatgctgggagaagcgcgctgctgtacgg
cgcgcaccgggtgcggagcccctcggcgagcgggtgaaacttctgtaatggcctgttcggtgcttttttatacggctgccagataaggcttcagc
atctgggcggctaccgctatgatcggggcgttcctgcaattcttagtgcgagtatctgaaaggggatacgcGCAGACCATGATCAGGGGGA
g
tttAAGTCTTCTTTCACGTGGC

-3'